

Decreased Neutrophil Function as a Cause of Retained Placenta in Dairy Cattle¹

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ABSTRACT

It is unclear why some cows fail to expel the placenta following calving. One theory suggests the fetal placenta must be recognized as “foreign” tissue and rejected by the immune system after parturition to cause expulsion of the placenta. We hypothesized that impaired neutrophil function causes retained placenta (RP). We examined the ability of neutrophils to recognize fetal cotyledon tissue as assessed by a chemotaxis assay, which utilized a placental homogenate obtained from a spontaneously expelled placenta as the chemoattractant. Neutrophil killing ability was also estimated by determining myeloperoxidase activity in isolated neutrophils. Blood samples were obtained from 142 periparturient dairy cattle in two herds. Twenty cattle developed RP (14.1%). Neutrophils isolated from blood of cows with RP had significantly lower neutrophil function in both assays before calving, and this impaired function lasted for 1 to 2 wk after parturition. The addition of antibody directed against interleukin-8 (IL-8) to the cotyledon preparation used as a chemoattractant inhibited chemotaxis by 41%, suggesting that one of the chemoattractants present in the cotyledon at parturition is IL-8. At calving, plasma IL-8 concentration was lower in RP cows (51 ± 12 pg/ml) than in cows expelling the placenta normally (134 ± 11 pg/ml). From these data, we suggest that neutrophil function is a determining factor for the development of RP in dairy cattle. Also, depressed production of IL-8 may be a factor affecting neutrophil function in cows developing RP. (**Key words:** chemotaxis, interleukin-8, periparturient dairy cow, retained placenta)

Abbreviation key: IL-8 = interleukin-8, ISU = Iowa State University Dairy Farm, NADC = National Animal Disease Center, NRP = nonretained placenta, RP = retained placenta.

INTRODUCTION

Retained placenta (RP) is a reproductive abnormality unique to the cow and water buffalo among domestic ruminants (Laven and Peters, 1996). Published reports on the incidence of RP range from 3 to 39% of parturitions; the incidence is somewhat influenced by the definition of RP utilized (Grunert, 1986, Laven and Peters, 1996). In a recent USDA study (NAHMS, 1996), the incidence of RP reported for dairy cows was $7.8 \pm 0.2\%$. Retained placenta causes significant economic losses, as many RP cows develop metritis and may suffer from infertility (Coleman et al., 1985), and RP also decreases milk production (Lucey et al., 1986). The cost per case was estimated to be \$285 (Guard, 1999). Both the mechanism of placenta expulsion and the cause of RP remain unclear. Various factors such as age, species, heredity, environment, hormones, and nutrition have been suggested as causes of RP (Hurley and Doane, 1989; Barnouin and Chassagne, 1991). However, data are conflicting, and no single endocrine or nutritional factor provides a satisfactory explanation for why cows develop RP. Gunnink (1984) proposed an intriguing theory that at parturition, when the blood supply to the placenta ceases, the placenta becomes a “foreign body.” He suggested that the maternal immune system must recognize and attack the fetal placenta as foreign to expel it. He demonstrated that cotyledons harvested from RP cows had less leukocyte chemoattractant than cotyledons harvested from cows that expelled the placenta normally after calving. He further demonstrated that leukocytes obtained from cows that would go on to develop RP were less able to recognize cotyledon tissue in a chemotaxis assay than leukocytes obtained from cows that would not develop RP. The lower chemotactic activity of leukocytes toward cotyledon superna-

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tant was seen before, at, and after calving in these landmark studies (Gunnink, 1984a, 1984b, 1984c). The lower amount of chemoattractant for leukocytes was confirmed by Heuwieser and Grunert (1987) in placentomes sampled at calving and 3 h after calving in RP cows. Romaniukowa (1984) demonstrated that neutrophils from cows that developed RP had a decreased response to chemoattractants and reduced ingestion capacity through the period 2 wk before and 2 wk after calving. Cai et al. (1994) demonstrated an association between lower neutrophil function and RP, but the association was significant only after parturition.

Before calving, lymphocytes may not be sensitive to placental antigens that prevent abortion of the fetus (Jiang and Vacchio, 1998; Rogers et al., 1998). However, the placenta is usually expelled within 3 to 8 h after parturition (Roberts, 1986). We felt that this was too short a time for lymphocytes to recognize the placenta as foreign and then mount a rejection process similar to those capable of rejecting an allogeneic graft. We chose to focus on the role innate immunity mediated by neutrophils might play in the recognition and rejection of the fetal placenta, because neutrophils can be more rapidly recruited and activated than lymphocytes. If decreased neutrophil function is a cause of RP, it should occur before parturition. To test our hypothesis, we bled cows frequently before calving to assess neutrophil function. We used an iodination assay to assess myeloperoxidase activity, which is a general index of oxygen-dependent killing capabilities of the neutrophils. We developed a chemotaxis assay with cotyledon supernatant as the source of chemoattractant for the neutrophils in an assay similar to that used by Gunnink (1984a, 1984b). In his original studies, Gunnink (1984) assessed chemotactic activity of a crude preparation of leukocytes (buffy coat). Our assay utilized isolated neutrophils only.

Interleukin-8 (IL-8), also known as neutrophil activating factor, is a cytokine produced by a wide variety of cell types in the presence of inflammatory stimuli such as lipopolysaccharide or Interleukin-1. Neutrophils are strongly attracted to areas of high concentrations of IL-8 (Elliott et al., 2000). We examined the role of IL-8 in the chemotaxis assays utilized in these studies and also determined plasma IL-8 concentrations in cows that did and did not develop RP.

MATERIALS AND METHODS

Animals

Dairy cattle ($n = 142$) from two herds were used for this study. Thirty-nine cows from the National Animal Disease Center (NADC) herd (Jersey and Holstein, 6 to 10 yr of age) and 103 dairy cattle at the Iowa State

University Dairy Farm (ISU) (65 cows, 38 heifers; primarily Holstein, with some Jersey, Ayrshire, Brown Swiss, Milking Short Horn, and Guernsey, 2 to 10 yr of age) were included.

Blood samples were collected by jugular venipuncture into tubes containing acid citrate dextrose for neutrophil isolation and into tubes containing sodium heparin for plasma isolation. Blood samples were taken from cows in the NADC herd from 2 wk before until 2 wk after calving. At the ISU herd, blood samples were taken from 2 wk before calving until the day after calving. Assays were performed on blood samples as described below.

Isolation of Neutrophils

Neutrophils were isolated from blood containing acid citrate dextrose by centrifugation as previously described (Roth and Kaerberle, 1981b). The cell number was adjusted with PBS to 5×10^7 neutrophils/ml for myeloperoxidase assay and 4×10^6 neutrophils/ml for chemotaxis assay. While not measured for every preparation, historically the neutrophil preparation is > 95% neutrophils, with eosinophils as the main contaminating cell type, and the cells are > 98% viable as assessed by markers of cell apoptosis.

Myeloperoxidase Activity Assays

The ability of cells to incorporate radioactive iodine (^{125}I) into trichloroacetic acid-precipitable material is a measure of myeloperoxidase activity and was determined as previously described (Roth and Kaerberle, 1981a). The assay was performed in duplicate on neutrophils stimulated with opsonized zymosan. The specific neutrophil-mediated incorporation of iodine into trichloroacetic acid-precipitable material was the difference between total iodine incorporated in test wells and the nonspecific adsorption of iodine as determined in wells prepared in the absence of neutrophils. The myeloperoxidase assay was performed three times per week. Blood samples from four nonpregnant cows were taken every day that the myeloperoxidase assay was performed and used as internal laboratory controls, based on the assumption that these four animals had relatively stable immune systems. Results for the myeloperoxidase assay of the pregnant cows are reported as a percentage of the response seen in these internal laboratory controls for each sampling day.

Assay of Chemotaxis Toward Cotyledon Chemoattractant

The chemotaxis assay was run every day on cows during the last week of gestation. For cows housed at

the NADC, the assay was also performed during the first 2 weeks of lactation.

The assay was carried out in a 48-well chemotaxis chamber (Neuro Probe, Gaithersburg, MD). Chemoattractant was prepared with cotyledons harvested from the spontaneously expelled placenta of a single cow. Cotyledons were homogenized with physiological saline (1:1) at 10,000 rpm with a POLYTRON PT 3000 homogenizer (Brinkmann, Westbury, NY). The homogenate was centrifuged for 10 min at 2800 rpm ($1300 \times g$). Supernatant was harvested and aliquots were frozen at -20°C until use. The wells in the bottom section of the chemotaxis chamber were filled with $25\ \mu\text{l}$ of cotyledon chemoattractant. The addition of $25\ \mu\text{l}$ formed a meniscus projecting above the plane of the chamber. A $10\text{-}\mu\text{m}$ thick polyvinyl pyrrolidone-free polycarbonate filter membrane (Osmonics, Livermore, CA) was placed on top of the meniscus over each well. The polycarbonate filter membrane had pores that were $5.0\ \mu\text{m}$ in diameter to allow chemoattractants to flow from the bottom chamber to the top. This size pore also allows neutrophils to cling to and pass through the pores where they can be enumerated. After applying the polycarbonate membrane, the top portion of the chemotaxis chamber was fastened to the bottom chamber and $50\ \mu\text{l}$ (4×10^6 neutrophils/ml) of neutrophil suspension freshly isolated from the blood of the cow was added to the top chamber. The chamber was incubated for 30 min at 39°C in a humidified CO_2 incubator to allow neutrophils time to react to the chemoattractant. The chamber was then disassembled and the side of the filter that had faced the upper chamber containing the neutrophil suspension was washed and wiped off three times with phosphate buffered saline to remove nonmigrated cells. The side of the filter that had faced the cotyledon suspension was stained (STAT STAIN, Volu-Sol, Inc., Salt Lake City, UT) and examined under oil immersion on a light microscope. The number of neutrophils that had migrated through the filter was quantified by counting the number of neutrophils present in five microscope fields at $1000\times$. Each sample was tested in duplicate. Results are reported as the average number of neutrophils observed per five fields in the duplicate wells.

Neutralization of Chemoattractant by Anti-IL-8 Antibody

If IL-8 is one of the chemoattractants present in the cotyledon supernatant, it should be possible to block the chemotactic response of the neutrophils using an antibody to bind the IL-8. Mouse antibody against ovine IL-8 (Serotec Ltd, Oxford, UK) is known to cross react with bovine IL-8 (Caswell et al., 1999) and was added

to cotyledon homogenate supernatant at a final concentration of 20 ng/ml. To avoid dilution of cotyledon supernatant by the addition of the antibody, freeze-dried cotyledon supernatant was reconstituted in the antibody solution to make up the same final concentration of cotyledon material as was used in the chemotaxis assays described above. Isotype-matched mouse antibody was also added to cotyledon homogenate at a final concentration of 20 ng/ml in control wells for comparison. Reconstituted cotyledon supernatant was gently mixed with anti-IL-8 antibody or isotype control for 30 min at 39°C before use. Neutrophils obtained from 26 healthy cows (lactating and nonlactating) were used in the top chambers of the wells for this assay. Each neutrophil isolate was assayed in duplicate anti-IL-8 antibody and duplicate isotype antibody wells.

Plasma Level of IL-8

Interleukin-8 was measured with an IL-8 ELISA kit (R&D Systems, Minneapolis, MN) designed to measure human plasma IL-8 concentrations. This kit has previously been validated for use in the bovine (Galligan and Coomber, 2000; Riollot et al., 2000). For this analysis, a subset of plasma samples taken from 7 RP cows and 7 NRP cows paired by date of calving were used. These cows were all from the NADC herd. Duplicate assay wells were used for each sample to provide a mean IL-8 concentration.

Statistical Analysis

To investigate the impact of RP on relevant variables, we performed split-plot analyses of variance. The statistical model included effects of disease (RP), time (days relative to parturition), and the interaction of disease and time (disease \times time). The mean square error for the cow within-disease was used as the error term to evaluate the effect of disease. Residual error [time \times cow within-disease] was used to evaluate the effect of repeated measures factor, time, and its interaction with disease (disease \times time). Differences were considered significant at $P < 0.05$.

RESULTS

Incidence of Retained Placenta

Cows were considered to have developed RP if the fetal membranes were retained more than 24 h postpartum. Twenty of 142 cows studied developed RP (14.1%), with 7 RP cases in the 39 cows at NADC (17.9%) and 13 RP cases in the 103 cows at ISU (12.6%). At ISU, there were 2 RP cases in the 38 heifers (5.3%) and 11 RP cases in the 65 cows (16.9%).

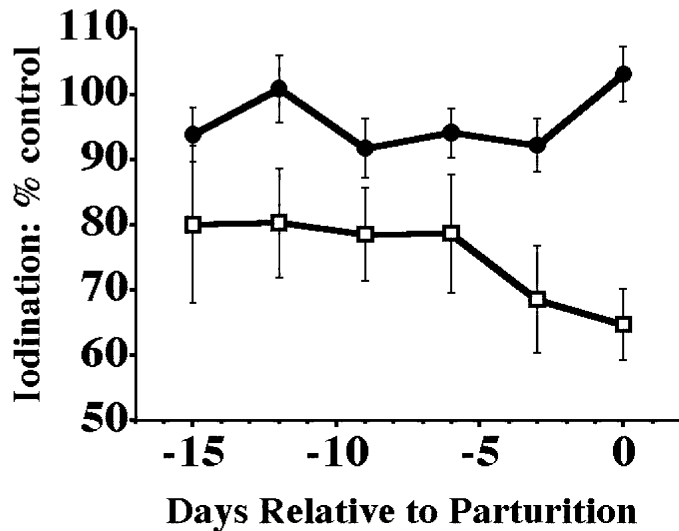


Figure 1. Myeloperoxidase activity of neutrophils in cows from both herds with retained placenta ($n = 20$, □) was significantly lower (disease effect, $P < 0.01$) than in cows without retained placenta ($n = 122$, ●) before parturition.

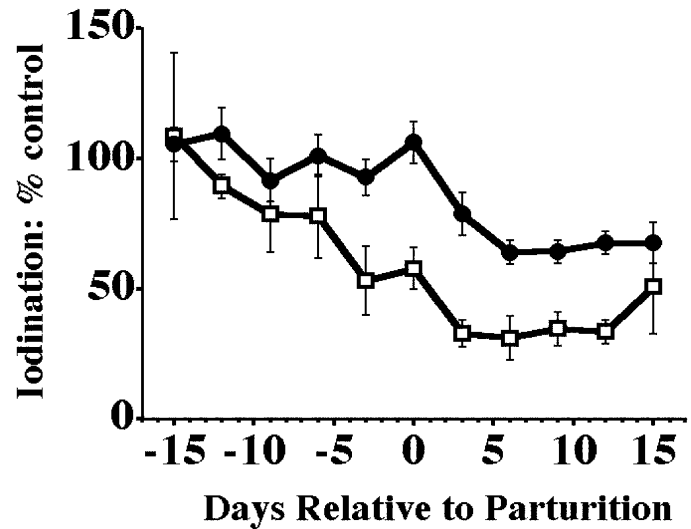


Figure 2. Myeloperoxidase activity of neutrophils in National Animal Disease Center cows with retained placenta ($n = 7$, □) was significantly lower (disease effect, $P < 0.01$) than in cows without retained placenta ($n = 32$, ●) before and after parturition.

Myeloperoxidase Activity

The myeloperoxidase activity in RP cows was significantly lower than in nonretained placenta (NRP) cows throughout the sampling period (Figure 1). Myeloperoxidase activity of neutrophils from NRP cows did not change before calving. However, myeloperoxidase activity of neutrophils from RP cows exhibited a gradual decrease as the time of calving approached and reached a nadir the day of calving. The effect of RP was significant ($P < 0.01$). The greatest difference was observed the day of calving (mean \pm SEM; $103 \pm 4.2\%$ in NRP cows vs. $64.7 \pm 5.4\%$ in RP cows). Examination of the data obtained from cows at NADC that were sampled both before and after calving reveals that myeloperoxidase activity in cows with RP remained significantly lower than in NRP cows the first 14 d of lactation as well. NRP cows at NADC did exhibit decreased myeloperoxidase activity after calving, but the activity was always significantly greater than in RP cows (Figure 2).

Chemotaxis Assays

Chemotactic activity, expressed as cell numbers per five microscopic fields, was also significantly lower in RP cows than NRP cows from 7 d before calving until 1 d after calving (disease effect; $P < 0.001$; Figure 3). This difference was greatest 1 d after calving (387 ± 23 cells/5 fields vs. 142 ± 30 cells/5 fields). This was primarily due to a sudden decrease in chemotactic activity in RP cows at calving. NADC cows were sampled in lactation as well, and NADC cows with RP exhibited

significantly lower levels of chemotactic activity than NADC cows with NRP for at least the first week of lactation (Figure 4).

In this assay, cotyledon material used as the chemotactant had been harvested from one particular cow. It is possible that the chemotactic properties of this preparation were somehow affecting whether cells from some cows responded and others did not. To check for an effect of the cotyledon preparation, cotyledon

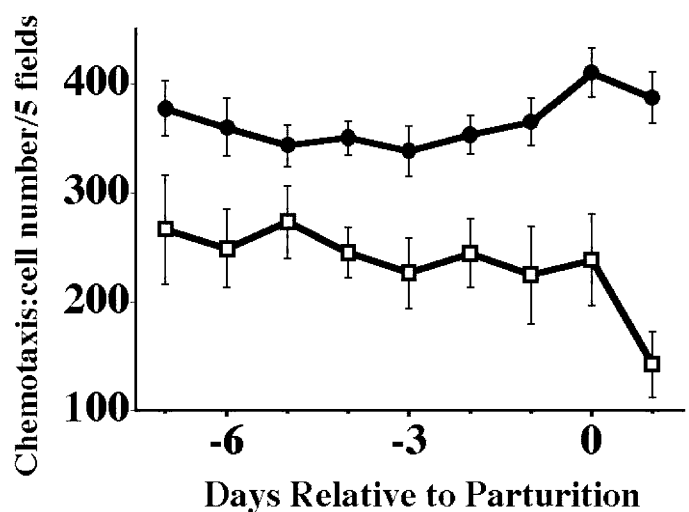


Figure 3. Chemotactic activity of neutrophils toward cotyledon supernatant in cows from both herds with retained placenta ($n = 20$, □) was significantly lower (disease effect, $P < 0.001$) than in cows without retained placenta ($n = 122$, ●) before parturition.

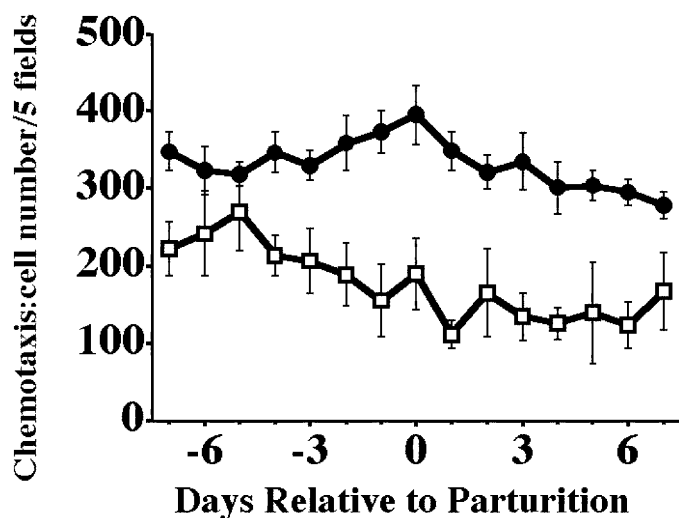


Figure 4. Chemotactic activity of neutrophils toward cotyledon supernatant in National Animal Disease Center cows with retained placenta ($n = 7$, □) was significantly lower (disease effect, $P < 0.001$) than in cows without retained placenta ($n = 32$, ●) before and after parturition.

material from two more spontaneously expelled placentas were homogenized and prepared for use in the chemotactic assay. Wells were prepared with these two new preparations and the preparation used in the chemotaxis assays described above. Neutrophils from six healthy nonpregnant, nonlactating cattle were isolated and used to assess their chemotactic activity toward these three cotyledon sources of chemoattractant. There was no significant difference among these three cotyledon supernatants in their ability to stimulate chemotaxis among the six neutrophil sources. The chemotaxis scores were 338 ± 49 , 360 ± 43 , and 416 ± 64 cells/5 fields for cotyledon preparations 1, 2, and 3, respectively. Cotyledon preparation #1 was the preparation used in the assays of RP and NRP cows.

Neutralization of Chemoattractant by Anti-IL-8 Antibody

The addition of an antibody that specifically binds IL-8 to the cotyledon chemoattractant preparation reduced chemotactic activity of the neutrophils by an average of 41% across the 26 pairs of wells prepared with the neutrophils of 26 different cows. There were 271 ± 18 cells/5 fields when cotyledon supernatant plus isotype control was used as the chemoattractant and 159 ± 10 cells/5 fields when cotyledon supernatant plus anti-IL-8 antibody was used as the chemoattractant ($P < 0.0001$).

Plasma Concentrations of IL-8

Concentrations of IL-8 in plasma were measured in a subset of 7 RP and 7 NRP cows from NADC. Plasma

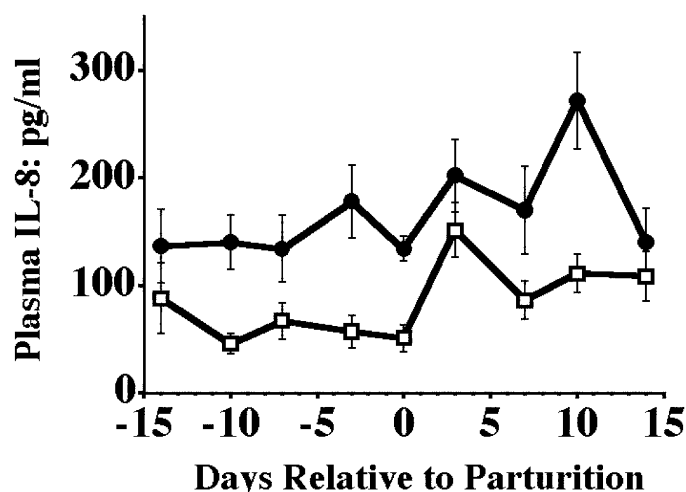


Figure 5. Concentration of interleukin-8 (IL-8) in plasma was significantly higher (disease effect, $P < 0.01$; disease \times time interaction effect, $P < 0.05$) in nonretained placenta ($n = 7$, ●) cows than in cows with retained placenta ($n = 7$, □) in the National Animal Disease Center herd.

concentrations of IL-8 throughout the periparturient period were lower in RP cows than in NRP cows (disease effect; $P < 0.01$, disease \times time effect; $P < 0.05$). Plasma concentrations of IL-8 on the day of calving were 134 ± 11 pg/ml for NRP cows and 51 ± 12 pg/ml for RP cows. Although this level increased after parturition in RP cows, it remained lower than the level observed in NRP cows from -14 to 14 d (Figure 5). Plasma IL-8 concentration in samples from six nonpregnant, nonlactating cows were also assayed for IL-8 and were found to be below the detection limit of the assay, which is 31 pg/ml.

DISCUSSION

Gunnink did the pioneering work establishing a relationship between RP and reduced chemotactic activity of leukocytes. He used the buffy coat harvested from centrifuged whole blood as a source of leukocytes and a cotyledon homogenate as the source of chemoattractant (Gunnink, 1984b, 1984c). We attempted to use his method but found that the buffy coat isolated from dry cows exhibited very poor chemotaxis toward cotyledon supernatant. Chemotactic assays are generally felt to be most appropriate for phagocytes, such as monocytes and neutrophils (Rice and Bognold, 1992; Wilkinson, 1998). While activated lymphocytes can be induced to be attracted to chemoattractants, the response generally requires more time (Poo et al., 1988; Hayashi et al., 1989). Gunnink incubated the buffy coat cells in his Boyden chamber apparatus for just 15 min before counting the cell number under a microscope. He found very few cells exhibiting chemotaxis compared with our re-

sults, even though he did not dilute the buffy coat cell preparation. He did not mention either the cell density of leukocytes in the buffy coats used or the kind of leukocytes involved in the chemotactic response. We speculate that his observations were made on monocytes in the buffy coat. Few neutrophils will exist in the buffy coat when prepared as is described in his paper. We found that isolated neutrophils responded very strongly toward cotyledon supernatant. Thus, we focused on neutrophil activity rather than mononuclear cells in this study and proved that lower chemotactic activity was occurring before calving in cows that would go on to develop RP. Similarly, myeloperoxidase activity, which we utilized as an index of general killing capability of the neutrophils, was markedly reduced before calving in those cows that would go on to develop RP. The work of Cai et al. (1994) demonstrated reduced myeloperoxidase activity and reduced chemotactic activity (using opsonized zymosan stimulated serum as the chemoattractant) in cows that developed RP, but the differences in neutrophil activity were apparent only after calving (Cai et al., 1994). There was no difference in RP and NRP cows before calving. They interpreted their data to suggest that the act of parturition initiated suppression of neutrophil function and that perhaps RP was the cause of the immune suppression that increased the susceptibility to metritis in cows. We sampled our cows more frequently and observed impairment in neutrophil function both before and after parturition. From our data, we suggest that decreased neutrophil function is not the result of RP but is likely the cause of RP. Romaniukowa (1984) compared neutrophil ingestion capacity in RP and NRP cows and reported lower activity during the 2 wk before and after calving in RP cows. He also reported reduced chemotactic activity in RP cows before calving. This study also suggests that a generalized loss of neutrophil function before calving places a cow at risk of developing RP.

Several other research groups (Heuwieser and Grunert, 1987; Hoedemaker et al., 1992) have also performed chemotactic assays using cotyledon preparations as the chemoattractant providing further evidence that the placenta attracts neutrophils. However, the chemoattractants contained in the cotyledon suspensions have not been identified. Interleukin-8 is a potent chemoattractant and activator of neutrophils. Interleukin-8 is secreted by monocytes, activated neutrophils, endothelial, and epithelial cells (Baggiolini et al., 1994; Caswell et al., 1999). Interleukin-8 is also secreted by the uterus and placenta of humans and the amount of IL-8 secreted increases at the end of gestation (Elliott et al., 2000; Laham et al., 1999). Interleukin-8 appears to initiate dilatation of the cervix, which plays an important role in the process of parturition in humans (Maehara et

al., 1996; Laham et al., 1999). We demonstrated that neutralization of IL-8 in cotyledon supernatant by antibody directed against IL-8 greatly reduced the chemoattractant properties of the cotyledon preparation. It did not completely eliminate the chemotactic response. Perhaps antibody neutralization was not complete. However, it seems more likely that other chemoattractants are also present in the cotyledon preparations. Interleukin-8 secreted by the cotyledon and/or uterus may be absorbed into the systemic circulation and act to recruit and activate neutrophils. Another possible role exists for IL-8, apart from its effect on neutrophils. Interleukin-8 is also known to increase collagenase secretion (Luo et al., 2000), which accelerates the separation of fetal cotyledon from maternal caruncle (Eiler and Hopkins, 1992). The observation of lower plasma IL-8 concentration in RP cows supports both these concepts.

It was recently demonstrated that there is less immunoglobulin in colostrum from cows with RP than in colostrum from NRP cows (Lona-D and Romero-R; 2001). The authors suggested that RP caused a reduction in lymphocyte abilities to produce immunoglobulin. However, it may be more likely that the lack of immunoglobulin in the colostrum of RP cows is further proof of an association between reduced immune function before calving and the subsequent susceptibility to RP.

The hypothesis that RP is caused by immune dysfunction at calving is a unifying theory that helps explain the epidemiological evidence that deficiency or excess of a variety of nutrients or hormones can affect the incidence of RP. This unifying theory suggests that those factors affect RP incidence because deficiency or excess of those factors impacts the immune system.

CONCLUSIONS

Cows developing RP have impaired neutrophil function, as assessed by chemotaxis toward cotyledon supernatant preparations and myeloperoxidase activity. The impaired neutrophil function is observed before calving, suggesting this is a cause of retained placenta rather than an effect of RP. One of the chemoattractants in the cotyledons that attracts the neutrophils is Interleukin-8. Cows that will go on to develop retained placenta have less IL-8 in their plasma before calving than cows that will expel the placenta normally.

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REFERENCES

- Baggiolini, M., B. Dewald, and B. Moser. 1994. Interleukin-8 and related chemotactic cytokines—CXC and CC chemokines. *Adv. Immunol.* 55:97–179.
- Barnouin, J., and M. Chassagne. 1991. An aetiological hypothesis for the nutrition-induced association between retained placenta and milk fever in the dairy cow. *Ann. Rech. Vet.* 22:331–343.
- Cai, T.-Q., P. G. Weston, L. A. Lund, B. Brodie, D. J. McKenna, and W. C. Wagner. 1994. Association between neutrophil functions and periparturient disorders in cows. *Am. J. Vet. Res.* 55:934–943.
- Caswell, J. L., D. M. Middleton, and J. R. Gordon. 1999. Production and functional characterization of recombinant bovine interleukin-8 as a specific neutrophil activator and chemoattractant. *Vet. Immunol. Immunopathol.* 67:327–340.
- Coleman, D. A., W. V. Thane, and R. A. Dailey. 1985. Factors affecting reproductive performance of dairy cows. *J. Dairy Sci.* 68:1793–1803.
- Eiler, H., and F. M. Hopkins. 1992. Bovine retained placenta: Effects of collagenase and hyaluronidase on detachment of placenta. *Biol. Reprod.* 46:580–585.
- Elliott, C. L., D. M. Slater, W. Dennes, L. Poston, and P. R. Bennett. 2000. Interleukin-8 expression in human myometrium: Changes in relation to labor onset and with gestational age. *Am. J. Reprod. Immunol.* 43:272–277.
- Galligan, C. L., and B. L. Coomber. 2000. Effects of human IL-8 isoforms on bovine neutrophil function in vitro. *Vet. Immunol. Immunopathol.* 74:71–85.
- Grunert, E. 1986. Etiology and pathogenesis of retained bovine placenta. Page 237–242 in *Current Therapy in Theriogenology*. Morrow D. A. Saunders Publishing, Philadelphia, PA.
- Guard, C. 1999. Set up fresh and milking cows for successful A. I. *Hoard's Dairyman*. 8–9.
- Gunnink, J. W. 1984a. Post-partum leucocytic activity and its relationship to caesarian section and retained placenta. *Vet. Q.* 6:55–57.
- Gunnink, J. W. 1984b. Pre-partum leucocytic activity and retained placenta. *Vet. Q.* 6:52–54.
- Gunnink, J. W. 1984c. Retained placenta and leukocytic activity. *Vet. Q.* 6:49–51.
- Hayashi, Y., A. Imaizumi, T. Yoshida, and M. Torisu. 1989. Staining of surface antigen on migration lymphocytes in the chemotaxis membrane I: complement C5 attracts helper/inducer T cell much more than suppressor/cytotoxic T cell and NK cell. *J. Exp. Pathol.* 4:181–192.
- Heuwieser, W., and E. Grunert. 1987. Significance of chemotactic activity for placental expulsion in cattle. *Theriogenology* 27:907–912.
- Hoedemaker, M., L. A. Lund, and W. C. Wagner. 1992. Function of neutrophils and chemoattractant properties of fetal placental tissue during the last month of pregnancy in cows. *Am. J. Vet. Res.* 53:1524–1529.
- Hurley, W. L., and R. M. Doane. 1989. Recent developments in the roles of vitamins and minerals in reproduction. *J. Dairy Sci.* 72:784–804.
- Jiang, S.-P., and M. S. Vacchio. 1998. Multiple mechanisms of peripheral T cell tolerance to the fetal “allograft”. *J. Immunol.* 160:3086–3090.
- Laham, N., S. P. Brennecke, and G. E. Rice. 1999. Interleukin-8 release from human gestational tissue explants: Effects of gestation, labor, and chorioamnionitis. *Biol. Reprod.* 61:823–827.
- Laven, R. A., and A. R. Peters. 1996. Bovine retained placenta: Aetiology, pathogenesis and economic loss. *Vet. Rec.* 139:465–471.
- Lona, V., and C. Romero. 2001. Low levels of colostral immunoglobulins in some dairy cows with placental retention. *J. Dairy Sci.* 84:389–391.
- Lucey, S., G. J. Rowlands, and A. M. Russell. 1986. Short-term associations between disease and milk yield of dairy cows. *J. Dairy Res.* 53:7–15.
- Luo, L., T. Ibaragi, M. Nozawa, T. Kasahara, M. Sakai, Y. Sasaki, K. Tanebe, and S. Saito. 2000. Interleukin-8 levels and granulocyte counts in cervical mucus during pregnancy. *Am. J. Reprod. Immunol.* 43:78–84.
- Maehara, K., N. Kanayama, E. E. L. Maradny, T. Uezato, M. Fujita, and T. Terao. 1996. Mechanical stretching induces interleukin-8 gene expression in fetal membranes: A possible role for the initiation of human parturition. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 70:191–196.
- Poo, W.-J., L. Conrad, and C. A. Janeway, Jr. 1988. Receptor-directed focusing of lymphokine release by helper T cells. *Nature (London)* 332:378–390.
- Rice, J. E., and L. P. Bognold. 1992. Chemotaxis of polymorphonuclear leukocytes in whole blood in the ‘spares-pore’ polycarbonate (Nucleopore) membrane/Boyden chamber assay. *J. Immunol. Methods* 149:121–125.
- Riollet, C., P. Rainard, and B. Poutrel. 2000. Differential induction of complement fragment C5a and inflammatory cytokines during intramammary infections with *Escherichia coli* and *Staphylococcus aureus*. *Clin. Diagn. Lab. Immunol.* 7:161–167.
- Roberts, S. J. 1986. Puerperal infections, uterine infections and diseases. Page 373–393 in *Veterinary Obstetrics and Genital Diseases*. 3. David & Charles Inc., Woodstock, NY.
- Rogers, A. M., I. Boime, J. Connolly, J. R. Cook, and J. H. Russell. 1998. Maternal-fetal tolerance is maintained despite transgene-driven trophoblast expression of MHC class I, and defects in Fas and its ligand. *Eur. J. Immunol.* 28:3479–3487.
- Romaniukowa, K. 1984. Phagocytosis in the uterus. Page 33–38 in *Proc. 10th Int. Congr. Anim. Reprod. Artificial Insemination*. University of Illinois, Champaign-Urbana, IL.
- Roth, J. A., and M. L. Kaeberle. 1981a. Evaluation of bovine polymorphonuclear leukocyte function. *Vet. Immunol. Immunopathol.* 2:157–174.
- Roth, J. A., and M. L. Kaeberle. 1981b. Isolation of neutrophils and eosinophils from the peripheral blood of cattle and comparison of their functional activities. *J. Immunol. Methods* 45:153–164.
- Wilkinson, P. C. 1998. Assays of leukocyte locomotion and chemotaxis. *J. Immunol. Methods* 216:139–153.